

Resolving a zoological mystery: the kouprey is a real species

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The kouprey is a rare and enigmatic forest ox discovered by scientists in Cambodia only in 1937. Numerous morphological hypotheses have been proposed for the origin of the kouprey: that it is a species closely related to banteng and gaur, two other wild oxen of southeast Asia; a morphologically divergent species placed in a separate genus, named *Novibos*; a wild species linked to aurochs and domestic cattle; a vicariant population of banteng; a feral cattle; or a hybrid of banteng with either zebu cattle, gaur or water buffalo.

In a recent paper, which gained a lot of media coverage, Galbreath *et al.* analysed mitochondrial DNA sequences and concluded that the kouprey never existed as a wild, natural species, and that it was a feral hybrid between banteng and zebu cattle.

Here we analyse eight DNA markers—three mitochondrial regions and five nuclear fragments—representing an alignment of 4582 nucleotides for the holotype of the kouprey and all related species. Our results demonstrate that the kouprey is a real and naturally occurring species, and show that Cambodian populations of banteng acquired a mitochondrial genome of kouprey by natural introgressive hybridization during the Pleistocene epoch.

Keywords: kouprey; *Bos sauveli*; *Bos javanicus*; introgression; hybridization; conservation

1. INTRODUCTION

The kouprey was described as a new species, *Bos sauveli* on the basis of a calf captured in Preah Vihear province of Cambodia and kept alive at the Vincennes Zoo near Paris until 1940 (Urbain 1937). In the middle of the twentieth century, its range was already limited to northern provinces of Cambodia, and slightly beyond the borders with Thailand, Laos and Vietnam (Sauvel 1949). Populations declined dramatically during the last five decades due to multiple possible factors including uncontrolled hunting, deforestation and competition with domestic livestock linked to human demographic growth, and wars. No sightings of kouprey have been reported by scientists since the 1980s, suggesting that the species is now extinct (MacKinnon & Stuart 1988). The animal is now the national emblem of Cambodia, and an icon of wildlife conservation in southeast Asia.

The kouprey is a mysterious animal with striking characters, including spectacular curving horns and a pronounced dewlap, a pendulous skin at the base of the neck that can nearly touch the ground in some older males. Numerous morphological hypotheses have been proposed for the origin of the kouprey: that it is a species closely related to banteng (*Bos javanicus*) and gaur (*Bos frontalis*), two other wild oxen of southeast Asia (Urbain 1937; Bohlken 1961; Pfeffer & Kim-San 1967); a morphologically divergent species placed in a separate genus, named *Novibos* (Coolidge 1940); a wild species linked to aurochs and domestic cattle (Pfeffer & Kim-San

1967; Groves 1981); a vicariant population of banteng (Corbet & Hill 1992); a feral cattle (Wharton 1957; Bohlken 1963); or a hybrid of banteng with either zebu cattle, gaur or water buffalo (Cheminaud 1939; Edmond-Blanc 1947; Bohlken 1958).

In 2004, the holotype of the kouprey (no. 1940–51, MNHN) was included in a molecular phylogeny of the tribe Bovini, and the results suggested close affinities with banteng and gaur (Hassanin & Ropiquet 2004). Seven nucleotide signatures were detected in the mitochondrial cytochrome *b* gene (*Cytb*) of the holotype. Surprisingly, four of these signatures were rediscovered in the sequences of Cambodian banteng, and Galbreath *et al.* (2006) concluded that the kouprey was not a valid species, but a feral hybrid resulting from a crossing between domestic zebu and wild banteng. Unfortunately, this conclusion gained a lot of media coverage (e.g. Bakalar 2006; Casey 2006; Derr 2006). Interviewed for CBS News, Galbreath said 'It is surely desirable not to waste time and money trying to locate or conserve a domestic breed gone wild. The limited funds available for conservation should be used to protect wild species' (Casey 2006).

We suggested, however, that Galbreath *et al.* (2006) misinterpreted the DNA data (Hassanin & Ropiquet 2007; see also Grigson 2007; Hedges *et al.* 2007). In the mitochondrial tree, here constructed with three different markers (*Cytb*, *CO2* and *D-loop*; figure 1), Cambodian banteng are indeed found to be closely related to the kouprey (mean distance: 1.4%) and more distant to gaur (5.0%), but they are unexpectedly found to be highly divergent from Javan banteng (5.4%). Particularly

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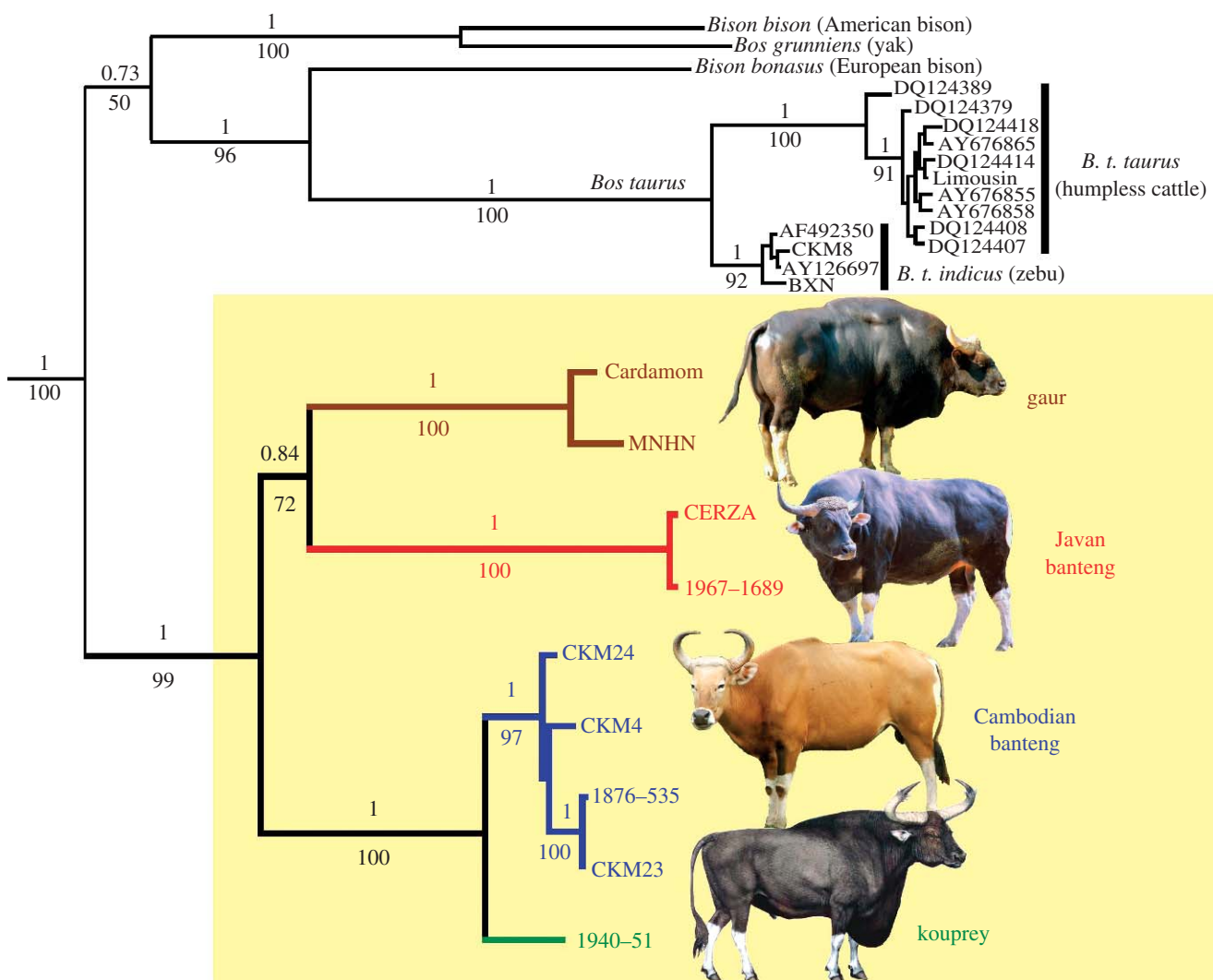


Figure 1. Phylogenetic position of Cambodian banteng using mitochondrial sequences from three markers (cytochrome *b*, *D-loop* and *CO2*). The values indicated above the branches are Bayesian posterior probabilities and those found below are bootstrap percentages calculated with the maximum-likelihood method. The photos are from Brent Huffman (Javan banteng, Ultimate Ungulate Images) and A.H. (Cambodian banteng and gaur). The illustration of kouprey is modified from Coolidge (1940).

relevant is the presence of a large insertion in the mitochondrial *D-loop* of Javan banteng (176 nt), which is not found in Cambodian banteng. Two conflicting hypotheses can therefore be proposed to interpret the mitochondrial data (figure 2). The first hypothesis assumes that the Cambodian and Javan banteng belong to two distinct species, and that the kouprey diverged morphologically from the former owing to hybridization with another species needing to be identified. This hypothesis is compatible with the conclusions of Galbreath *et al.* (2006) if we accept that the hybridization of banteng occurred with zebu. The second hypothesis recognizes the kouprey as a valid species, and implies the existence of a mitochondrial introgression event, in which the mitochondrial genome of kouprey was transferred into the ancestor of Cambodian banteng by natural hybridization. Both hypotheses are supported by the fact that viable and potentially fertile hybrids have been produced in captivity between various species of the genus *Bos* (Van Gelder 1977).

The aim of the present study was to reject one of the two hypotheses in order to conclusively define the taxonomic status of the kouprey. The fact that the

mitochondrial genome is maternally inherited limits its application to the evolutionary study of maternal lineage. For this reason, we also sequenced five non-coding nuclear fragments for the holotype of the kouprey and all living species of oxen, bison and yak: two fragments of the Y-chromosome were used to trace the evolutionary history of paternal lineage, and three independent autosomal genes were analysed to evidence possible cases of inter-specific hybridization.

2. MATERIAL AND METHODS

(a) Taxonomic sample

All seven species of the subtribe Bovina (Hassanin & Ropiquet 2004; Wilson & Reeder 2005) are represented in this study (table 1): (i) *Bos sauveli*, with the holotype of the kouprey (No. 1940–51, MNHN), (ii) *Bos javanicus*, with four Cambodian banteng and two Javan banteng, (iii) *Bos taurus*, with two humplless domestic cattle (subspecies *B. taurus taurus*) and two southeast Asian zebu (subspecies *B. taurus indicus*), (iv) *Bos frontalis*, with two different populations of gaur, (v) *Bos grunniens* (yak), (vi) *Bison bison* (American bison), and (vii) *Bison bonasus* (European bison).

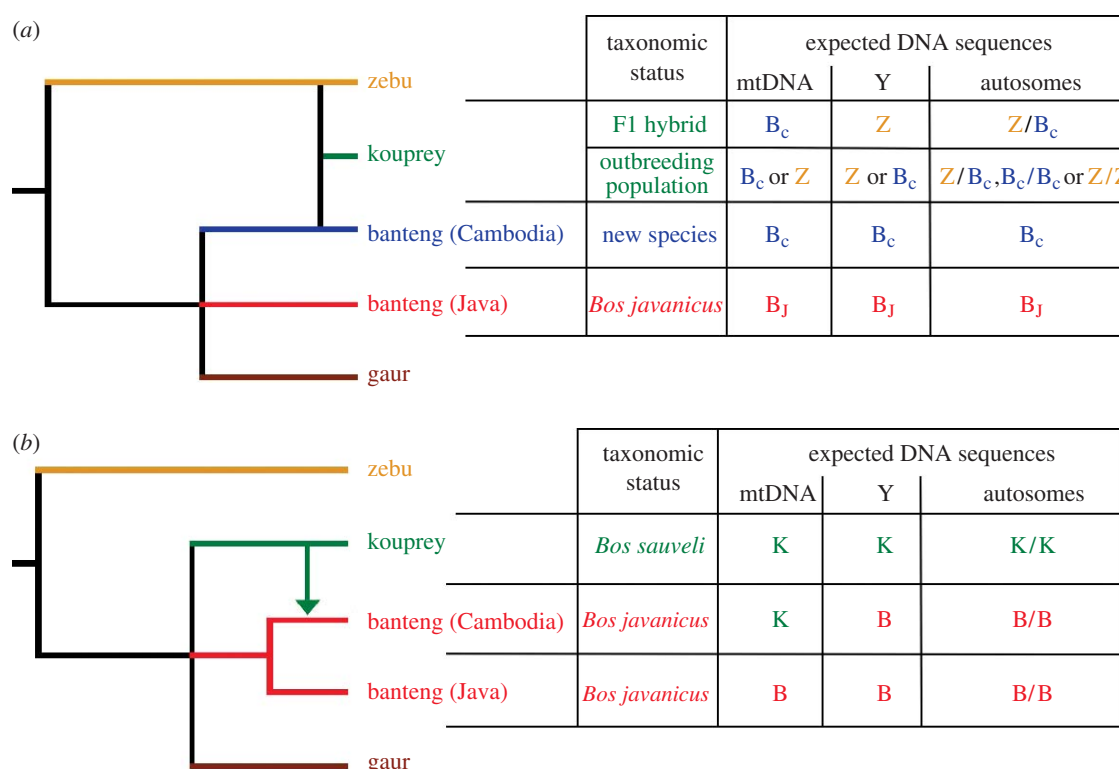


Figure 2. Two conflicting hypotheses for explaining the close relationship between mitochondrial sequences of kouprey and Cambodian banteng. (a) Hypothesis 1. Hybrid origin of the kouprey: banteng \times zebu. (b) Hypothesis 2. Mitochondrial introgression into the ancestor of Cambodian banteng.

(b) Molecular markers

The nuclear genes were chosen, firstly, because our preliminary analyses revealed that banteng and zebu differ at several nucleotide sites, and, secondly, because they are unlinked markers, with different locations in the genome of *B. taurus*: chromosome 11 for intron 1 of the beta-spectrin non-erythrocytic 1 gene (*SPTBN1*); chromosome 14 for intronic and exonic regions of the thyroglobulin gene (*TG*); chromosome 17 for intron 7 of the beta-fibrinogen gene (*FGB*); and the Y-chromosome for two non-coding fragments of the sex-determining region Y (*SRY*). BLAST searches performed on the assembled genome of *B. taurus* indicate that all four nuclear genes are present in single copy, therefore, avoiding PCR amplification of paralogous sequences and facilitating the phylogenetic interpretations.

Three mitochondrial regions were also analysed: the 5' part of the control region (also named *D-loop*), and two protein-coding genes, i.e. the complete cytochrome *b* (*Cytb*) and subunit II of the cytochrome *c* oxidase (*CO2*).

(c) DNA extraction, amplification and sequencing

Total DNA was extracted from fresh tissues (blood, muscle or hairs) or from bones of specimens conserved in the MNHN collections as detailed in Hassanin & Ropiquet (2004). The standard PCR conditions were as follows: 3 min at 94°C; 30–40 cycles of denaturation/annealing/extension with 45 s at 94°C for denaturation, 45 s at 50–60°C for annealing and 1 min at 72°C for extension; and 7 min at 72°C. For DNA extracted from museum specimens, several sets of primers were designed for amplifying and sequencing overlapping PCR products. For DNA extracted from fresh tissues, PCR amplifications were done using external primers only. The protein-coding mitochondrial genes (*Cytb* and *CO2*) were amplified using published primers (Hassanin & Ropiquet 2004). The 5' part of the *D-loop*

region was obtained using the two following primer pairs: (i) 5'-ACT-AAT-ACC-AAC-AGC-CGG-CAC-3' (F: forward) and 5'-GAG-TAC-AAA-GTC-TGT-GTT-GAG-3' (R: reverse) and (ii) 5'-TAG-TTC-CAC-AAA-CGC-AAA-GAG-C-3' (F) and 5'-GTT-GCT-GGT-TTC-ACG-CGG-CAT-GG-3' (R). Intron 1 of *SPTBN1* was amplified using the three following primer pairs: (i) 5'-AGT-GCA-GCC-TTG-AAA-GGT-AC-3' (F) and 5'-CAA-AGT-TCA-CTG-CCC-AAC-ATA-AGC-3' (R), (ii) 5'-CCC-TTC-AGT-ACC-CAA-GTG-CTAC-3' (F) and 5'-CAA-ACT-TAG-AAC-AAA-TAT-CTG-CAC-3' (R), and (iii) 5'-GCT-CTC-TTG-GCT-TTC-ACA-CCT-G-3' (F) and 5'-ACA-CCC-CTG-TTC-TCG-TTC-CTA-G-3' (R). The *TG* fragment was amplified using the three following primer pairs: (i) 5'-GAG-CCC-AAG-AAA-TGT-GAG-TC-3' (F) and 5'-AGC-CTG-CCC-ATC-ACT-AAA-TC-3' (R), (ii) 5'-GAC-AAC-AGC-TGG-TCT-CAA-CTG-C-3' (F) and 5'-GAC-CAA-GAT-GCA-TAT-GTG-CTA-AG-3' (R), and (iii) 5'-CCC-CTT-TGC-CAG-TCC-ATG-GAG-TG-3' (F) and 5'-GTG-CTG-GGA-TTG-GAG-ACC-AGG-GTC-3' (R). Intron 7 of *FGB* was amplified with the four following primer pairs: (i) 5'-CCA-CAA-CRG-CAT-GTT-CTT-CAG-CAC-3' (F) and 5'-AGA-GCT-TAG-TAC-AGT-GCT-GGC-A-3' (R), (ii) 5'-CAT-GAC-AGC-AAA-CAT-GAC-TAG-TGA-C-3' (F) and 5'-GCT-TTC-ATA-TCT-CTT-AGT-GTT-ACA-G-3' (R), (iii) 5'-CAA-CTG-TAA-TTT-GAG-CAC-AGT-TAC-3' (F) and 5'-AGC-AAG-AAA-GGG-AGA-TGA-CTG-G-3' (R), and (iv) 5'-GAA-TAT-TGG-TGA-ATT-TGC-CAC-ATG-3' (F) and 5'-CAA-GTT-AAT-TCT-TTG-CAA-AGC-CCA-C-3' (R). The first non-coding fragment of *SRY*, located upstream of the 5' end of the coding sequence (*SRY-5'*), was amplified with 5'-CCT-GTT-AAG-TAG-CTT-TGC-TTG-AG-3' (F) and 5'-CAC-AGC-TGG-ACT-GTA-AAC-ATC-GT-3' (R). The second non-coding fragment of *SRY*, located downstream of the 3' end of the coding

Table 1. Origin of the DNA sequences.

taxa	N ^o /origin	sex	Cytb 1140 nt	CO2 582 nt	D-loop 710 nt	FGB 554 nt	SPTBN1 442 nt	TG 627 nt	SRY-5' 224 nt	SRY-3' 303 nt
<i>Bos saureli</i> kouprey	1940–51 (holotype) MNHN	M	AY689189 ^a	AY689202 ^a	EF693812 ^b	EF693828 ^b	EF693843 ^b	EF693858 ^b	EF693871 ^b	EF693888 ^b
<i>Bos javanicus</i> Cambodian banteng	CKM4 Teuk Chhou zoo	M	EF685912 ^c	EF685920 ^c	EF693806 ^b	EF693822 ^b	EF693837 ^b	EF693852 ^b	EF693867 ^b	EF693878 ^b
	CKM24 Phnom Tamao zoo	M	EF685914 ^c	EF685922 ^c	EF693808 ^b	EF693824 ^b	EF693838 ^b	EF693853 ^b	EF693868 ^b	EF693879 ^b
	1876–535 MNHN	M	EF693796 ^b	EF693800 ^b	EF693804 ^b	EF693825 ^b	EF693840 ^b	EF693855 ^b	EF693869 ^b	EF693880 ^b
	CKM23 Phnom Tamao Zoo	F	EF685913 ^c	EF685921 ^c	EF693807 ^b	EF693823 ^b	EF693839 ^b	EF693854 ^b	—	—
<i>Bos javanicus</i> Javan banteng	CERZA Hermival-les-Vaux 1967–1689 MNHN	M	AY689188 ^a	AY689201 ^a	EF693809 ^b	EF693827 ^b	EF693842 ^b	EF693857 ^b	EF693870 ^b	EF693881 ^b
<i>Bos taurus taurus</i> domestic cattle	NCBI database	F	EF693797 ^b DQ124418 ^d	EF693801 ^b DQ124418 ^d	EF693805 ^b DQ124418 ^d	EF693826 ^b BGP ^e	EF693841 ^b BGP ^e	EF693856 ^b BGP ^e	— AB039748 ^f	— AB039748 ^f
<i>Bos taurus indicus</i> zebu	Limousin France BXN Vietnam	M M	EF693798 ^b EF693799 ^b	EF693802 ^b EF693803 ^b	EF693815 ^b EF693816 ^b	EF693829 ^b EF693830 ^b	EF693844 ^b EF693845 ^b	EF693859 ^b EF693860 ^b	EF693872 ^b EF693873 ^b	EF693882 ^b EF693883 ^b
<i>Bos frontalis</i> gaur	CKM8 Cambodia Cardamom Cambodia	M M	EF685907 ^c EF685910 ^c	EF685915 ^c EF685918 ^c	EF693817 ^b EF693814 ^b	EF693831 ^b EF693833 ^b	EF693846 ^b EF693848 ^b	EF693861 ^b EF693865 ^b	EF693874 ^b EF693875 ^b	EF693884 ^b EF693885 ^b
	Jardin des Plantes MNHN	F	AY689187 ^a	AY689199 ^a	EF693813 ^b	EF693832 ^b	EF693847 ^b	EF693862 ^b	—	—
<i>Bos grunniens</i> yak	Jardin des Plantes MNHN	M	NC_006380 ^g	NC_006380 ^g	NC_006380 ^g	EF693834 ^b	EF693849 ^b	EF693866 ^b	EF693876 ^b	EF693886 ^b
<i>Bison bison</i> American bison	Vincennes Zoo MNHN	M	AF036273 ^h	AY689197 ^a	EF693810 ^b	EF693835 ^b	EF693850 ^b	EF693863 ^b	EF693877 ^b	EF693887 ^b
<i>Bison bonasus</i> European bison	OSEB MNHN	F	AY689186 ^a	AY689198 ^a	EF693811 ^b	EF693836 ^b	EF693851 ^b	EF693864 ^b	AY079142 ⁱ	AY079142 ^j

^a Hassanin & Ropiquet (2004). ^b This study. ^c Hassanin & Ropiquet (2007). ^d H. D. Shin & L. H. Kim (2007, unpublished data). ^e <http://www.hgsc.bcm.tmc.edu/projects/bovine/>. ^f Kato *et al.* (1995). ^g A. San, W. Wang & L. Yu (2007, unpublished data). ^h Hassanin & Douzery (1999). ⁱ Verkaar *et al.* (2004).

	Y gene		autosomal genes [chromosome No, <i>B. taurus</i>]			
	<i>SRY</i> -5'	<i>SRY</i> -3'	<i>FGB</i> [17]	<i>SPTBN1</i>	<i>TG</i> [14]	
	1112	1123	1113355	2333344	22222334444444556	
	13793590	2253820	67883483735	2792557902	12388846779562344489350	
	48523584	14619043	144157154361	8640099579	81545622346029212977601	
Taxa						
<i>Bos sauveli</i> 1940-51	ACAACCGT	ACGTACTT	GCTAGGCTGATC	GTCCCTGCCTC	GGCGCTAGAT	GCGAGCGGAAGAC
<i>Bos javanicus</i> CKM4	---GT---	---*G-CC	---A---	A-T---	---T-A-A-	A-A-GG---
<i>Bos javanicus</i> CHM24	---GT---	---*G-CC	---A---	A-T---	---T-A-A-	A-A-GG---
<i>Bos javanicus</i> 1876-535	---GT---	---*G-CC	---M---	A-T---	---T-A-A-	A-A-GG---
<i>Bos javanicus</i> CKM23	????????	????????	---A---	A-T---	---T-A-A-	A-A-GG---
<i>Bos javanicus</i> CERZA	---GT---	---*G-CC	---G---	A-T---	---T-A-A-	A-A-GG---
<i>Bos javanicus</i> 1967-1689	????????	????????	---R??	A-T---	---T-A-A-	A-A-GG---
<i>Bos taurus taurus</i> Limousin	---GT---	---*G-CC	A*C---A-GA-G	---T-AT-AT	---CT-RY	---R-T
<i>Bos taurus taurus</i> NCBI	---GT---	---*G-CC	A*C---A-GA-G	---T-AT-AT	A---CT-AT	---A-T
<i>Bos taurus indicus</i> BXN	---GT---	---*G-CC	A*Y---R-GA-G	---T-AT-AT	A---CT-AT	---R-R-T
<i>Bos taurus indicus</i> CKM8	---GT---	---*T-C	A*---GA-GG	---T-AT-AT	---CT-AT	---T-AG-C
<i>Bos frontalis</i> Cardamom	AG-G--A-	G--*---	---A---	A---R---	AT***-T-	---T-G-MY
<i>Bos frontalis</i> MNHN	????????	????????	---A---	AG---	---CT---	---A-A-G---
<i>Bos grunniens</i>	G-GGTT-	G--*---C	---A---	---G-T-	---CT---	---A*----
<i>Bison bison</i>	G-GT--C	---A*---C	---G---GA-	---T-	---MT---	---R-R-Y
<i>Bison bonasus</i>	G-GT--C	---A*---C	---G---GA-	---T-	---TT---	---G---

Figure 3. List of the 61 variable sites between nuclear sequences of *Bos* and *Bison* species. The five non-coding nuclear fragments include two regions of the *SRY* gene in the Y-chromosome (*SRY*-5' and *SRY*-3') and introns of three independent autosomal genes (*FGB*, *SPTBN1* and *TG*). Heterozygous nucleotide sites are highlighted in yellow. Cambodian *B. javanicus* sequences are labelled in blue and Javan sequences in red.

sequence (*SRY*-3'), was amplified with the following two sets of primers: (i) 5'-CAT-GTA-AAG-AAT-TCA-GAC-TTT-CC-3' (F) and 5'-CCA-TCT-AAC-TAA-TGA-CCA-ATC-TC-3' (R) and (ii) 5'-CTG-CTT-GAG-TTC-AAA-GAT-CAT-C-3' (F) and 5'-AGG-GAG-CTT-TCC-ATC-CAA-GTA-C-3' (R). Both strands of all PCR products were sequenced by Genoscreen (Lille, France) with the BigDYE TERMINATOR V. 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). Sequences generated for this study are available from the GenBank/EMBL/DBJ databases under accession numbers specified in table 1.

(d) Phylogenetic analyses

The mitochondrial tree was constructed under MrBAYES v. 3.1.2 (Huelsenbeck & Ronquist 2001), by applying the model of sequence evolution selected by MrMODELTEST v. 2.2 (Nylander 2004), i.e. GTR+I+G. Five Markov chains were run for 2 000 000 generations and sampled every 100 generations after an initial burn-in period of 20 000 cycles. The node robustness was estimated firstly by the bayesian posterior probabilities and, secondly, by the bootstrap percentages obtained with the maximum-likelihood method under PHYML v. 2.1b1 (Guindon & Gascuel 2003) after 1000 replicates.

In order to use three calibration points (see details below) we aligned 31 mitochondrial sequences, corresponding to the 16 specimens listed in table 1, as well as the following additional specimens: eight humpless domestic cattle (DQ124379, DQ124408, DQ124414, DQ124407, AY676855, AY676865, AY676858 and DQ124389); two zebu cattle (AY126697 and AF492350); three species of Bubalina, i.e. *Syncerus caffer* (Cytb, AF036275; CO2, U18825 and D-loop, EF693818), *Bubalus bubalis* (NC_006295) and *Bubalus depressicornis* (Cytb, AF091632; CO2, U18822 and D-loop, EF693819); and two out-group species, i.e. *Boselaphus tragocamelus* (Cytb, AJ222679; CO2, U62566 and D-loop, EF693820) and *Tetracerus quadricornis* (Cytb, AF036274; CO2, AY689196 and D-loop, EF693821).

(e) Molecular dating

Mitochondrial sequences were used for estimating divergence times using the relaxed Bayesian molecular clock method implemented in MULTIDIVTIME (Thorne & Kishino 2002).

The expected number *a priori* of time units between tip and root (rttm) was set at 14 Myr ago, with a standard deviation of 7 Myr ago. The Markov chains were sampled 10 000 times every 100 generations and the 'burn-in' period was set at 100 000 generations. Three calibration points were used for the analyses: the first two correspond to independent domestications of humpless cattle and zebu cattle between 8000 and 10 000 years BP (Loftus *et al.* 1994), and the third refers to the diversification of the subtribe Bovina (*Bos* and *Bison*), estimated between 3.89 and 5.53 Myr ago (Hassanin & Ropiquet 2004).

3. RESULTS AND DISCUSSION

(a) Analyses of nuclear sequences

Five non-coding nuclear fragments, including introns of three independent autosomal genes (*FGB*, *SPTBN1* and *TG*) and two regions of the *SRY* gene in the Y-chromosome, were sequenced for the holotype of the kouprey and all living species of oxen, bison and yak. What were the expected results with nuclear data (figure 2)? If the holotype of the kouprey was an F1 hybrid between Cambodian banteng (*B_c*) and zebu (*Z*), its Y-chromosome would be identical to that of zebu, while its autosomal genes would be heterozygous *Z/B_c*, and therefore characterized by several dimorphic nucleotide sites. If the holotype of the kouprey were a member of an outbreeding population, its autosomal loci would be either heterozygous *Z/B_c* or homozygous (*B_c/B_c* or *Z/Z*), and its Y-chromosome would be identical to that of zebu or banteng. As explained previously, the hypothesis of a hybrid origin for the kouprey argues that the Cambodian and Javan banteng belong to two different species. As a consequence, banteng from Cambodia and Java are expected to have different nuclear sequences (*B_c* and *B_j* alleles; figure 2). By contrast, if we accept the hypothesis of mitochondrial introgression, banteng from Cambodia and Java would share identical or highly similar nuclear sequences, while the kouprey would differ, at least at some loci, from all other species of the genus *Bos*.

Sixty-one nucleotide sites were found to be variable between nuclear sequences of *Bos* and *Bison* species (figure 3). The results provide conclusive proof for the existence of *B. sauveli*. Firstly, Cambodian and Javan

banteng share identical nuclear alleles, indicating that both populations belong to the same species, *B. javanicus*. Secondly, the sequences of the kouprey holotype do not contain heterozygous sites, which would be expected in the case of hybrid origin, and they differ from those found in other species of *Bos* (with the exception of the *FGB* gene, for which kouprey, banteng and gaur share the same allele). Thirdly, three nuclear sites are diagnostic for the holotype of the kouprey (figure 3): A in position 92 of *SRY-5'*; T in position 51 of *SRY-3'*; and G in position 262 of *TG*. The analysis of nuclear data demonstrates therefore that *B. sauveli* and *B. javanicus* are two distinct and valid species, and that the Cambodian banteng acquired a mitochondrial genome from the kouprey by introgressive hybridization.

(b) *Introgression of the mitochondrial genome of kouprey into the common ancestor of Cambodian banteng*

One fundamental question for the conservation of wild populations was to determine whether hybridization between kouprey and banteng occurred as a consequence of human activities. Using a relaxed molecular clock, we estimated that the hybridization occurred during the Pleistocene epoch, at 1.34 ± 0.45 Myr ago. As this estimate largely predates the origin of agriculture and domestication of plants and animals, it can be concluded that it was not a consequence of human intervention. The mitochondrial introgression supposes that at least one kouprey female, which was probably young in order to overcome inter-specific ethological barriers (Kendrick *et al.* 1998), was adopted into a herd of banteng. The event may have happened in open, dry, deciduous forests of Northern Cambodia, where several field biologists have reported the existence of temporary mixed herds between banteng and kouprey individuals (Edmond-Blanc 1947; Wharton 1957; Pfeffer 1969). The preservation of this unique habitat is crucial for keeping hope of conserving the kouprey and many other threatened species, such as banteng, gaur, wild water buffalo, Eld's deer, Asian elephant, tiger and leopard.

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